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1

2 **Transcriptional response of ovine lung to infection with jaagsiekte sheep retrovirus**

3

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12

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23 **Abstract**

24 Jaagsiekte sheep retrovirus (JSRV) is the etiologic agent of ovine pulmonary adenocarcinoma
25 (OPA), a neoplastic lung disease of sheep. OPA is an important economic and welfare issue for
26 sheep farmers and a valuable naturally-occurring animal model for human lung adenocarcinoma.
27 Here, we used RNA sequencing to study the transcriptional response of ovine lung tissue to
28 infection by JSRV. We identified 1,971 ovine genes differentially-expressed in JSRV-infected
29 lung compared to non-infected lung, including many genes with roles in carcinogenesis and
30 immunomodulation. The differential expression of selected genes was confirmed using
31 immunohistochemistry and RT-qPCR. A key finding was the activation of anterior-gradient-2,
32 yes-associated protein-1 and amphiregulin in OPA tumor cells, indicating a role for this
33 oncogenic pathway in OPA. In addition, there was differential expression of genes related to
34 innate immunity including genes encoding cytokines, chemokines and complement system
35 proteins. In contrast, there was little evidence for upregulation of genes involved in T-cell
36 immunity. Many genes related to macrophage function were also differentially expressed,
37 reflecting the increased abundance of these cells in OPA-affected lung tissue. Comparison of the
38 genes differentially regulated in OPA with transcriptional changes occurring in human lung
39 cancer revealed important similarities and differences between OPA and human lung
40 adenocarcinoma. This study provides valuable new information on the pathogenesis of OPA and
41 strengthens the use of this naturally occurring animal model for human lung adenocarcinoma.

42

43 **Importance**

44 Ovine pulmonary adenocarcinoma is a chronic respiratory disease of sheep caused by jaagsiekte
45 sheep retrovirus (JSRV). OPA is a significant economic problem for sheep farmers in many
46 countries and is a valuable animal model for some forms of human lung cancer. Here, we
47 examined changes in host gene expression that occur in the lung in response to JSRV infection.
48 We identified a large number of genes with altered expression in infected lung, including factors
49 with roles in cancer and immune system function. We also compared the data from OPA to
50 previously published data from human lung adenocarcinoma and found a large degree of overlap
51 in the genes that were dysregulated. The results of this study provide exciting new avenues for
52 future studies of OPA and may have comparative relevance for understanding human lung
53 cancer.

54
55 **Introduction**

56 Ovine pulmonary adenocarcinoma (OPA) is an infectious lung disease of sheep that is a
57 significant economic problem and welfare concern for sheep producers in many countries (1, 2).
58 The main clinical features of OPA are loss of condition and respiratory distress and in many
59 advanced cases there is an accumulation of fluid in the lungs that drains from the sheep's nostrils
60 when its head is lowered (3). Lung function is often further compromised by the presence of
61 bacterial or parasitic co-infection (1). By the time clinical signs become apparent, tumor growth
62 is typically extensive and the disease is invariably fatal.

63
64 OPA is caused by jaagsiekte sheep retrovirus (JSRV), an exogenous betaretrovirus (4). Despite
65 its etiological role in OPA, infected sheep produce only a limited adaptive immune response to

66 JSRV antigens and this has precluded the development of an effective preclinical serological
67 diagnostic test or vaccine to control the spread of disease (2). The mechanism underlying the
68 poor immune responsiveness of sheep to JSRV is not completely understood. It appears likely
69 that it is largely due to immunological tolerance elicited by the developmental expression of
70 closely related endogenous JSRV (enJSRV) proteins in the ovine fetal thymus (5, 6). However,
71 local immunomodulatory mechanisms are also proposed to contribute (7).

72

73 JSRV has a specific tropism for differentiated epithelial cells of the distal lung and OPA tumor
74 cells predominantly express markers of type II alveolar epithelial cells (AEC2) (8-10). An
75 unusual feature of JSRV is that the Env glycoprotein functions as a viral oncoprotein to drive
76 neoplastic transformation *in vitro* (11, 12) and *in vivo* (13-16). JSRV Env expression activates a
77 number of signaling pathways that control cellular proliferation, including PI3K-Akt and MEK-
78 ERK1/2 (17, 18). Several cellular factors that bind Env have been identified and are proposed to
79 be involved in transformation (19-21) but further work is necessary to provide a complete model
80 for Env-mediated tumorigenesis and to explain how this leads to the unique clinical presentation
81 of OPA.

82

83 In addition to its veterinary importance, OPA represents a valuable animal model for some forms
84 of human lung cancer due to similarities in histological appearance and the activation of common
85 oncogenic signaling pathways (22-25). In its early stages; such as in subclinical natural disease
86 and in experimentally-infected lambs, OPA resembles a minimally invasive adenocarcinoma
87 with a predominantly lepidic growth pattern (22, 24). In advanced natural disease, OPA is more
88 closely similar to adenocarcinoma with papillary or acinar predominant growth with or without

89 mucinous features (22, 25). The similarity of OPA to human lung adenocarcinoma suggests that
90 this naturally occurring sheep tumor could be valuable for understanding lung carcinogenesis,
91 particularly at the early stages of disease, which are difficult to diagnose and study in humans.

92

93 In order to examine the pathogenesis of OPA, we determined changes in host gene expression in
94 the lungs of lambs following experimental infection with JSRV. Many genes were identified to
95 have altered expression and we confirmed the upregulation of some of these using

96 immunohistochemistry and RT-qPCR. We also compared the differential gene expression of

97 OPA-affected animals with previously published data on the two most common types of non-

98 small-cell lung carcinoma (NSCLC) in humans; lung adenocarcinoma (LUAD) and lung

99 squamous cell carcinoma (LUSC). Collectively, this study provides new information that greatly

100 enhances our understanding of the host response to JSRV and provides a number of exciting new

101 avenues for future work on OPA.

102

103 **RESULTS**

104

105 **JSRV-induced gene expression in sheep lung tissue.**

106 Whole transcriptome profiling (RNA-Seq) was performed on lung samples derived from JSRV

107 infected (n=4) and mock-infected (n=4) specific pathogen-free (SPF) lambs. The samples used

108 were obtained from a previous study (9) in which 6-day old lambs were infected with JSRV by

109 intratracheal injection and euthanized when signs of respiratory distress appeared (66 – 85 days

110 post-inoculation). Mock-infected lung tissue came from age and sex-matched control lambs that

111 had been inoculated with cell culture medium (9). OPA tumor lesions were observed in

112 hematoxylin and eosin-stained lung tissue sections from infected lambs and confirmed with
113 immunohistochemistry (IHC) for JSRV Env (SU) protein. Such lesions were not present in lung
114 tissue from mock-infected lambs (9). Infected tissues contained many tumor foci distributed
115 throughout the lung that otherwise appeared histologically normal, a pattern typical of
116 experimentally-induced OPA (22). Samples for RNA-Seq were generated by pooling tissue
117 from 7 distinct sites of each lung. In order to capture specimens that were representative of the
118 whole tissue the samples analyzed were not specifically enriched for tumor cells.

119

120 RNA-Seq generated over 60 million reads per sample, of which approximately 80% mapped
121 uniquely onto the ovine and JSRV genomes (Table 1). A total of 15,149 sheep genes were
122 identified and reads were quantified to identify those that were differentially expressed between
123 mock-infected and JSRV-infected samples. Principal component analysis of the normalized
124 counts of sheep genes clearly separated the mock-infected and the JSRV-infected groups (Fig.
125 1A). JSRV infection produced a radical change in sheep gene expression with 1,971
126 differentially expressed transcripts identified between the two groups (Fig. 1B) (1237
127 upregulated and 734 downregulated). We defined differentially expressed genes as those
128 showing up or down regulation following JSRV infection with a false discovery rate (FDR)
129 below 0.05, regardless of the observed fold-change. The complete lists of mapped genes and
130 differentially expressed genes are presented in Supplementary Dataset S1. Hierarchical
131 clustering of all differentially expressed genes is shown in Fig. 1C. Fig. 1D summarizes the 25
132 most significantly upregulated and downregulated genes.

133

134 Although the RNA-Seq analysis revealed clear differences in gene expression between the
135 infected and control groups, one infected lamb (Infected_F_85days) showed an intermediate
136 overall expression pattern (Fig 1A, 1C). Of the four infected lambs, this animal had the lowest
137 percentage of reads mapping to JSRV in the RNA-Seq data (Table 1) and therefore likely had the
138 smallest proportion of tumor-affected tissue and the largest contribution from ‘healthy’
139 uninfected tissue. Nevertheless, hierarchical clustering grouped this animal with the other
140 infected lambs (Fig. 1C, D) and so it was included in subsequent analyses.

141

142 **Functional analysis of RNA-Seq data.**

143 KEGG pathway enrichment analysis of the upregulated and downregulated genes was performed
144 using the DAVID annotation software (<https://david.ncicrf.gov/> (26)) (Supplementary Dataset
145 S2). Upregulated pathways in the OPA-affected lung included genes involved in metabolic
146 pathways, epithelial cell differentiation, cell cycle and wound healing. Pathways involved in
147 immune response and inflammation were down-regulated. Ingenuity Pathway Analysis (IPA)
148 (27) was also used to functionally analyze the list of differentially expressed genes. The main
149 ‘Diseases and Biofunctions’ related to the differentially expressed genes are shown in Fig. 2. As
150 with DAVID analysis, cancer related functions, such as cell proliferation and tissue
151 development, were identified as highly enriched whereas those related to immune response and
152 inflammation were less strongly represented. These results revealed key signaling networks in
153 cancer, including neovascularization, cell viability and tumor growth, to be activated in JSRV-
154 infected tissue compared to mock-infected tissue (Fig. 3). Based on these analyses, we used IHC
155 to validate selected upregulated markers, focusing in particular on pathways of relevance to OPA
156 pathogenesis, as discussed below.

157

158 **Cancer-related gene expression in OPA-affected lung tissue.**

159 Many of the most significantly upregulated genes in OPA have previously been associated with
160 important aspects of tumorigenesis in mice and humans. These include functions such as cell
161 proliferation and differentiation (*CLDN2*, *CTSL*, *PROM2*, *AGR2*, *EPHA7*, *SOX9*, *KRT18*),
162 angiogenesis (*MMP9*, *CCLA2*, *EPHB2*, *CXCL8*), metastasis (*CCLA2*, *EPHB2*, *AGR2*, *HS6ST2*,
163 *MUC1*) and signal transduction, including EGF family members (*AREG*, *EREG*, *NRG2*) and Wnt
164 signaling components (e.g., *WNT10B*, *PPARG*, *KRT18*, *LEF1*, *AXIN2*, *FZD3*, *FZD5*, *ROR1*,
165 *CTNNB1* and *CDH1*). In addition, factors previously identified as specific markers of lung
166 adenocarcinoma were upregulated, including *PROM2*, *CLDN3* and *TJP3*, as were genes
167 proposed to have potential diagnostic or prognostic value in human cancers, including *MMP9*,
168 *AGR2*, *SULF1*, *NHSL1*, *LGR5*, *MUC1* and *PIK3C2G*.

169

170 Although several factors related to angiogenesis were found to be upregulated using RNA-Seq, a
171 previous study reported that vascular endothelial growth factor (VEGF) signaling was
172 downregulated in OPA (28). The RNA-Seq data is consistent with that study, finding reduced
173 expression of *VEGFD*, *VEGFA* and *VEGFR2* (*KDR*) in JSRV-infected lambs, although only
174 *VEGFD* passed our cut-off for statistical significance. The upregulation of *MMP9* in OPA-
175 affected lung tissue found by RNA-Seq is also in agreement with the previous study (28).

176

177 The upregulation of *AGR2* (anterior gradient-2) was of particular interest as this protein has been
178 shown to promote oncogenesis in adenocarcinomas of several tissues, including the lung (29).
179 To analyze the expression of *AGR2* in OPA, IHC was performed on lung tissue sections from

180 natural and experimental OPA and mock-infected lambs. The cytoplasm of bronchiolar
181 epithelial cells of mock-infected lung labeled strongly for AGR2 but there was no labeling of
182 cells in the alveolar compartment (Fig. 4A, D). In contrast, in JSRV-infected lung, there were
183 many regions of strong AGR2 labeling and these correlated closely with areas of JSRV-Env
184 labeling in serial adjacent sections (Fig. 4B, C, E, F). This was evident even on relatively small
185 tumor foci suggesting that AGR2 is activated early following JSRV infection.

186 Immunofluorescent labeling confirmed the co-expression of JSRV Env and AGR2 in infected
187 lung tissue, although not every infected cell cluster labeled positively for AGR2 (Fig. 4G-J).

188 AGR2 has been shown to stimulate the expression of the EGFR ligand amphiregulin (*AREG*) in
189 adenocarcinoma cells (30). *AREG* was also upregulated in OPA and, as for AGR2, IHC
190 demonstrated positive labeling for *AREG* in OPA tumor cells from both natural and
191 experimentally-induced disease (Fig. 4K-M).

192

193 The activation of *AREG* by AGR2 is mediated by Yes-associated protein (YAP1), a nuclear
194 effector of the Hippo signaling pathway (30), which led us to ask whether Hippo signaling is
195 involved in OPA. YAP1 was not found to be differentially expressed by RNA-Seq but to
196 examine this question further, IHC was performed on tissue sections of mock-infected and OPA-
197 affected lung using antibodies to the phosphorylated form of YAP1 and to total YAP1 (Fig. 5).

198 IHC detected both the phosphorylated and unphosphorylated forms of YAP1 in the bronchiolar
199 epithelium and in the alveolar compartment of mock-infected lung (Fig. 5A, D). As expected,
200 the phosphorylated (inactive) form was predominantly detected in the cytoplasm, whereas an
201 antibody to total YAP1 (which detects active and inactive forms) exhibited prominent nuclear
202 labeling that was more intense than labeling of the cytoplasm. Tumor cells in experimental OPA

203 had strong nuclear labeling for total YAP1 (Fig. 5B) but this appeared less intense in natural
204 OPA (Fig. 5C). IHC for regulators of the Hippo pathway, MST1/2 and LATS1/2, found these
205 proteins were readily detectable in AEC2s in healthy sheep lung (Fig. 5G, J), as previously
206 reported in mouse lung (31). OPA tumor cells also labeled strongly for both markers (Fig. 5H, I,
207 K, L). Interestingly, the distribution of labeling for LATS1/2 differed between experimentally-
208 induced tumors and natural cases of OPA, being predominantly cytoplasmic in experimental
209 disease and predominantly nuclear in natural disease. While a complete analysis of the
210 activation state of the Hippo pathway is outside the scope of this study, these data indicate that
211 the AGR2-YAP1-AREG axis is active in OPA and may contribute to oncogenesis in this disease.

212

213 **Immune response to JSRV infection and oncogenesis.**

214 The RNA-Seq analysis identified altered expression of many genes related to immune responses
215 including various cytokines and chemokines (e.g., *CSF2*, *CCL2*, *CXCL6*, *CXCL8*, *CXCL14*,
216 *IL1A*, *IL6*, *TGFB3* and *TNFSF18* were upregulated and *CSF1*, *CCL4*, *CCL18*, *CCL26*, *CXCL12*,
217 *IL15* and *TNF* were downregulated), along with several members of the complement pathway
218 (*C3*, *C5*, *C6*, *C7*, *MBL1* (*ENSOARG00000010165*), *CFI*, *CD46*, *PTX3*, *C4BPA*, *C4BPB*
219 upregulated and *CIQA* and *CIQB* downregulated), confirming a substantial host response to
220 infection and tumor growth. Selected cytokines were also evaluated by RT-qPCR in
221 experimentally-induced and natural OPA and this confirmed their differential expression (Fig.
222 6). Interestingly, the expression data does not support strong induction of type I interferon
223 responses in JSRV-infected lung. Although IFNA was not identified using RNA-Seq, RT-qPCR
224 analysis found it to be downregulated in natural OPA (Fig. 6). Expression of IFNB1 was not
225 significantly changed in JSRV-infected tissue. Furthermore, when considering a recently

described panel of 90 highly conserved mammalian type-1 interferon-stimulated genes (32), only one (IRF7) was significantly upregulated in our data.

IFNG expression was not significantly upregulated in cases of experimental OPA suggesting little or no activation of cellular Th1 responses in early disease. However, RT-qPCR found that IFNG was increased in tissue from cases of advanced natural OPA (Fig. 6B), consistent with a previous report of IFNG-positive macrophages in natural OPA (7). Markers of T-lymphocytes were either not significantly changed (CD3 (gamma, delta and epsilon chains) and CD4) or downregulated (CD8, alpha and beta chains). Interestingly, expression of *FOXP3* and *CD44*, both markers of regulatory T-cells (Tregs), was significantly increased in OPA. Tregs negatively regulate T-cell immune responses through a number of mechanisms and have been shown to reduce the ability of CD8 T-cells to produce TNF (33). Consistent with this, *TNF* expression was downregulated in experimentally and naturally-infected animals (Fig. 6).

In agreement with previous studies (7, 34), macrophages were observed in many sections of the experimental OPA cases studied here. In addition, approximately 10% (198 of 1971) of the differentially expressed genes in our analysis have macrophage-related functions (Fig. 7; Supplementary Dataset S3), confirming that macrophage-related gene expression is altered in JSRV-infected tissue. Upregulated genes include some previously associated with an inflammatory phenotype (e.g., *HIF1A*, *IL1A*, *CXCL8*, *CSF2*, *IRAK1*, *IRF7*) or an immunoregulatory phenotype (e.g., *CD163*, *CCL2*, *LGMN*, *MMP9*, *MMP14*), suggesting that a complex pattern of macrophage activation exists in OPA. Selected macrophage markers were also studied by IHC. Labeling for CD68, a commonly-used marker of monocytes and tissue

249 macrophages, identified cells located in normal alveolar lung tissue and along the margin of
250 tumor foci (Fig. 8A-C). In comparison, an antibody to CD163, a marker for macrophages with
251 an immunomodulatory phenotype (35), labeled many more cells, including cells that surrounded
252 or infiltrated the tumor foci (Fig. 8D-F). Macrophages in or around OPA foci also labeled
253 positively for legumain (LGMN), another marker associated with an immunoregulatory
254 phenotype (Fig. 8G-I) and hypoxia-inducible factor 1-alpha (HIF1A, which is associated with an
255 inflammatory macrophage phenotype (Fig. 8J-L). LGMN was also detected in alveolar
256 macrophages in mock-infected tissue, while HIF1A labeling in mock-infected lung was limited
257 to the bronchiolar epithelium. Interestingly, the antibodies to HIF1A and LGMN also labeled
258 some tumor cells whereas anti-CD163 and anti-CD68 did not. Consistent with the greater
259 abundance of macrophages in OPA-affected lung, RNA-Seq identified increased expression of
260 several myeloid cell chemoattractants (e.g., *CCL2*, *CSF2*, *S100A8*, *S100A9*, and *CXCL8*) in
261 JSRV-infected tissues.

263 **Comparison of gene expression of ovine pulmonary adenocarcinoma with human lung**
264 **adenocarcinoma.**

265 We next compared the RNA-Seq data from OPA with previously published expression data from
266 the two most common types of NSCLC (LUAD and LUSC). Gene counts data from RNA-Seq
267 studies on tumors from patients with LUAD and LUSC were downloaded from The Cancer
268 Genome Atlas (TCGA) database (portal.gdc.cancer.gov) (36). Data were selected from patients
269 for whom matched normal and tumor data were available (57 LUAD and 49 LUSC;
270 Supplementary Dataset S4 shows their clinical annotation). The genes differentially expressed
271 between tumor and normal samples were identified in each clinical disease stage (LUAD I – IV

272 and LUSC I - III); (Supplementary Dataset S5). To compare differentially expressed genes in
273 sheep and human cancers, we considered only those sheep genes with one-to-one orthologues in
274 humans (1726 out of 1971 (87%)). We then compared the lists of differentially expressed
275 orthologous genes between OPA and the different stages of human LUAD and LUSC by
276 calculating the Pearson correlation coefficient between the log ratios of genes in common
277 between the two lists. The correlation plot of orthologous genes between OPA and the different
278 stages of human LUAD and LUSC (Fig. 9A) revealed that the gene expression profile of OPA is
279 more similar to LUAD than it is to LUSC and that OPA is more closely correlated with stage I
280 LUAD than with later stages. The main Diseases and Biofunctions associated with the
281 differentially expressed orthologous genes in OPA and stage I LUAD were identified with IPA
282 (Fig. 9B). Supplementary Dataset S6 lists the fold-change of the genes differentially expressed
283 in OPA with their human orthologues in LUAD and LUSC and identifies those genes with
284 similar or divergent changes in expression between the two species. Fig. 9C summarizes the
285 fold-change of the 50 genes most significantly deregulated in OPA with their human orthologue
286 from stage I LUAD. Of these, 37 were consistent in their direction of change and 13 were
287 discordant.

288

289 **DISCUSSION**

290 The pathogenesis of OPA is intriguing. How does JSRV infection and expression of its
291 oncogenic Env protein elicit the striking clinical and pathological phenotype typical of this
292 disease? As the mechanisms underlying this process remain largely undetermined, here we used
293 a global RNA-Seq approach to gain insight into the gene pathways and networks that are altered
294 during the early stages of OPA. We identified 1971 differentially expressed sheep genes and

295 validated several of these by IHC or RT-qPCR. Some changes revealed the altered cellular
296 composition of infected and uninfected lung. For example, the increased number of
297 macrophages and the presence of tumor cells derived from AEC2s in infected tissue is reflected
298 in the upregulation of markers of these cell types. However, many of the genes and pathways
299 that have altered expression in JSRV infection have not been previously studied in OPA but are
300 associated with important aspects of OPA pathogenesis including tumor pathways and local
301 immune responses. Collectively, this study provides important new information on the host
302 response to JSRV infection that will be valuable for studies of OPA directly and for exploitation
303 of the sheep disease as a lung cancer model.

304

305 In this study, we utilized tissue from experimentally-infected SPF lambs in order to avoid the
306 potential confounding effects of additional respiratory infections that commonly occur in natural
307 cases of OPA (1). For welfare reasons, experimentally-infected lambs must be culled when
308 respiratory signs first appear and therefore the tissues studied represent an early stage of disease
309 compared to the advanced stage of natural cases of OPA. We used sections of total lung tissue
310 because we were interested in analyzing the gene expression of the whole infected tissue. A
311 consequence of this approach is that the experimental OPA samples contained a greater
312 proportion of histologically normal lung tissue than tumor tissue. We anticipated that this excess
313 of 'healthy' tissue would reduce the sensitivity of our study to detect differentially expressed
314 genes, in particular down-regulated genes (37, 38). This would appear to be the case as only four
315 genes were found to have downregulated expression by more than two-fold in OPA.
316 Nevertheless, while this approach is expected to underestimate the fold-changes of differentially-
317 expressed genes it should still provide the correct order of significance. Future work will focus

318 on analysis of gene expression in specific cell types such as tumor cells and myeloid cells, which
319 should increase the sensitivity of detection of differentially expressed genes in those cell subsets.

320

321 **Oncogenic signaling pathways in OPA.**

322 The mechanisms involved in JSRV Env-mediated tumorigenesis are not completely understood
323 and multiple cellular pathways appear to be activated (15, 17, 18). Several studies have shown
324 that Env expression leads to activation of the PI3K/Akt and MAPK/ERK1/2 signaling pathways
325 (14, 18, 39, 40), while additional pathways including EGFR and Wnt signaling may also play a
326 role (15, 19, 41, 42). The RNA-Seq data provided evidence supporting a variety of changes in
327 gene expression related to carcinogenesis, including increased expression of several ligands of
328 EGFR and other ERBB family receptors and upregulation of a variety of ligands and receptors
329 related to Wnt signaling in JSRV-infected lung tissue.

330

331 Interestingly, the major components of the PI3K/Akt and MAPK/ERK1/2 signaling pathways
332 were not significantly upregulated. Indeed, DAVID pathway analysis reported that both of these
333 pathways were downregulated in OPA. There are several possible explanations for this apparent
334 discrepancy. For example, activation of the MAPK and PI3K/Akt pathways is mediated by
335 protein phosphorylation cascades, which may not correspond directly to changes in transcription
336 of those genes. In addition, while previous studies have shown roles for Akt and ERK signaling
337 in tumor cells or transfected cell lines, the RNA-Seq analysis presented here reflects global
338 transcriptional changes within the whole tissue, which comprises multiple cell types. Most
339 importantly, the MAPK and PI3K/Akt pathways are involved in many cellular processes in
340 addition to oncogenesis, including immune responses (43, 44), and the list of genes that DAVID

341 and similar tools uses for identifying members of a pathway is very broad. For example, several
342 of the genes identified by DAVID as involved in the PI3K/Akt and MEK/ERK1/2 pathways are
343 involved in immune responses (e.g., *TLR2*, *CSF1*, *COL6A3*, *NFKB1* and *TNF*), which is
344 consistent with the other changes in immune and inflammatory responses observed in our study.

345

346 An important novel finding in our study was the activation of AGR2 in OPA tumor cells (Fig. 4).
347 AGR2 is a protein disulfide isomerase localized in the endoplasmic reticulum in normal cells,
348 but is upregulated in a variety of human adenocarcinomas where it may also be present in
349 secreted and cell-surface-bound forms (45, 46). AGR2 is associated with poor prognosis in
350 several cancer types and appears to mediate its oncogenic effect through the regulation of other
351 genes including *TP53* (47) and *AREG* (30) and through extracellular functions such as promoting
352 angiogenesis and extracellular matrix remodeling (46, 48). In humans, AGR2 expression is
353 significantly higher in LUAD than in LUSC (49). The activation of AREG by AGR2 has been
354 shown to be dependent on the Hippo pathway effector protein YAP1 in human adenocarcinoma
355 cell lines (30). Consistent with this, IHC analysis confirmed the presence of nuclear YAP1 in
356 OPA tumor cells (Fig. 5), suggesting a possible role for Hippo signaling in OPA pathogenesis.
357 While further work is required, the transcriptional upregulation of AGR2, EGFR ligands and
358 other oncogenic factors identified by RNA-Seq provides new opportunities for understanding
359 oncogenic signaling in OPA. In turn, OPA provides a model for studying the function of these
360 pathways in a naturally occurring tumor, particularly at the early stages of tumorigenesis.

361

362 **Local immune responses in OPA-affected lung tissue.**

363 The absence of a significant adaptive immune response to JSRV in sheep is commonly attributed
364 to enJSRV expression in the fetal thymus during immune development, which is proposed to
365 lead to immune tolerance through the deletion of T-lymphocytes that recognize the closely
366 related JSRV proteins (5). The results of the RNA-Seq analysis suggest that JSRV infection
367 induces substantial immunological changes in lung tissue, including altered expression of
368 numerous cytokines, chemokines and complement factors together with an increase in
369 macrophages associated with tumor foci. These changes suggest that local immune-modulatory
370 mechanisms active within the OPA-affected lung might also suppress the immune response to
371 JSRV.

372

373 The activation of complement factors and complement regulatory factors in OPA is consistent
374 with a previous microarray analysis of gene expression in a mouse model of JSRV Env-mediated
375 transformation that also found evidence of complement upregulation (50). Complement has
376 been shown previously to inhibit infection of a number of viruses including retroviruses (51),
377 and studies on human and murine cancers have established roles for complement in modulating
378 tumor growth (52-55). Therefore, complement might also play an immunomodulatory role in
379 OPA tumors.

380

381 Macrophages exhibit significant functional plasticity (56, 57) and gene expression studies
382 suggest the existence of numerous subpopulations of macrophages, including some with
383 important roles in cancer (56-58). In addition, studies of mouse and human cancers have shown
384 that macrophages and other myeloid cells in the tumor microenvironment are essential for tumor
385 survival and metastasis (58, 59). Tumor-associated macrophages are also abundant in OPA (7)

386 but the phenotype of these cells and their functional significance is unclear. In experimentally-
387 induced OPA, we identified changes in the expression of 198 genes related to macrophage
388 function, including genes previously associated with an inflammatory or immunoregulatory
389 phenotype. Interestingly, the expression of macrophage markers in OPA appears to vary
390 depending on their location within the affected tissue, with cells on the periphery of tumor foci
391 being CD68-positive and cells within the tumor being CD163-positive and CD68-negative (Fig.
392 8). CD163 is regarded as a reliable marker for immunomodulatory macrophages that are
393 associated with poor prognosis in human tumors (60, 61). The positive CD163 labeling of OPA-
394 associated macrophages therefore suggests that these cells might also promote tumor growth in
395 OPA.

396

397 In contrast, the transcriptome analysis provided little evidence to support a strong adaptive
398 immune response in OPA. For example, there was no activation of T-cell markers other than a
399 modest but statistically significant increase in markers of Tregs (FOXP3 and CD44; although
400 note that CD44 is also a marker for macrophages and AEC2s). In addition, there was no
401 evidence of substantial activation of type 1 interferon responses in OPA, whereas IFNG
402 expression was not significantly changed in JSRV-infected lambs but was upregulated in natural
403 cases studied by RT-qPCR (Fig. 6). This is possibly due to the presence of concurrent bacterial
404 infections in the natural cases but demonstrates an important difference between early
405 experimental OPA and advanced natural cases.

406

407 Collectively, the gene expression data reported here suggest the presence of an
408 immunomodulatory environment within the OPA lung, which has the potential to suppress the

409 immune response to JSRV and tumor cells and to actively promote tumor growth. A more
410 detailed quantitative analysis of the role of tumor-associated myeloid cells, complement and
411 Tregs in OPA is necessary to determine their contribution to tumor growth and development of
412 clinical disease. This could reveal insights into the relevance of the tumor microenvironment to
413 the apparent immune tolerance of sheep to JSRV and may inform the design of vaccine strategies
414 for controlling OPA.

415

416 **Comparison of the transcriptomes of OPA and human LUAD.**

417 As OPA is frequently cited as an animal model for human lung adenocarcinoma, we compared
418 the transcriptome data from OPA with published data on human lung tumors in an attempt to
419 identify the similarities and differences between the diseases in the two species. The results
420 indicate closer similarity of the transcriptome of OPA with human LUAD than with LUSC and
421 in particular with early (stages I and II) compared to more advanced disease (stages III and IV)
422 (Fig. 9A, C; Supplementary Dataset S6). This is consistent with the histological appearance of
423 OPA which resembles a minimally invasive adenocarcinoma in its early stages (2, 22, 24, 25). It
424 would be interesting in future studies to compare gene expression in the early stage of disease
425 studied here with that of more advanced OPA.

426

427 Many genes showed a common pattern of differential expression in LUAD, LUSC and OPA but
428 there were also many that did not. Some of these reflect differences between LUAD and LUSC
429 and highlight the diversity of gene expression in the different tumor types. For example, 142
430 genes were upregulated in both OPA and stage I LUAD but downregulated or not significantly
431 altered in stage I LUSC. Notably, this includes *AGR2* and the Wnt pathway effector protein

432 beta-catenin. Similarly, 86 genes were upregulated in OPA and stage I LUSC but not
433 upregulated in stage I LUAD. In addition, there were 134 genes upregulated in OPA that were
434 downregulated in both LUAD and LUSC. These include *SFTPC* and *LAMP3* which are known
435 markers of AEC2s in sheep and humans (62). *LAMP3* has been detected in OPA previously but
436 is rarely expressed in human tumors except in bronchioloalveolar-adenocarcinoma (lepidic-
437 prominent adenocarcinoma-in-situ) (62), which were not represented in the LUAD cases in the
438 TCGA data studied. Interestingly, the upregulation of complement factors observed in OPA was
439 not evident in either LUAD or LUSC and several other key immune response related genes, such
440 as *TLR10*, *CCL4*, *CCL26*, *CD8A*, *CD8B*, *TNF* and *IDO1*, were specifically downregulated in
441 OPA but not in human LUAD, suggesting that innate immune responses in the sheep and human
442 diseases may differ in important ways. Collectively, this analysis provides support for the
443 similarity of experimental OPA to early stage LUAD but highlights that there remain many
444 differences between the sheep and human diseases.

445
446 In summary, the findings from this first large-scale analysis of host gene expression in OPA
447 significantly increase our understanding of the disease pathogenesis at a transcriptional level and
448 will inform future research directed at improving OPA disease control. Moreover, the
449 interspecies comparative data between sheep and humans provide additional support for the use
450 of OPA as a model for early stages of LUAD, particularly non-invasive forms. Finally, a deeper
451 understanding of the pathological changes of early tumors could help to identify novel
452 biomarkers for the early detection of cancer lesions in both species.

453

454 MATERIALS AND METHODS

455

456 **Animals and tissues.**

457 Tissues were available from a previous study in which four specific pathogen free (SPF) lambs
458 were inoculated through intra-tracheal injection with JSRV at six days of age (9). Four
459 additional lambs received cell culture supernatant (mock-infected control). Each group
460 contained one female and three male lambs. All lambs were caesarean-derived and housed in
461 SPF conditions to minimize the risk of acquiring additional respiratory infections. Once the
462 clinical signs of respiratory disease were apparent in the JSRV-infected animals (66d, 71d and
463 85d (n=2) post inoculation), lambs were culled. Each time a JSRV-infected lamb was
464 euthanized, a healthy animal from the mock-infected control group was culled to provide age and
465 sex-matched control tissues. Tissues were collected from 24 locations in each lung and stored in
466 liquid nitrogen for RNA extraction and in 10% buffered formalin for IHC. To study tissues from
467 animals with natural disease, lung samples were taken from four farm-raised adult sheep in the
468 advanced stages of clinical OPA and four clinically healthy adult sheep. Cases were selected
469 which had no gross appearance of bacterial or parasitic infection. All protocols involving animal
470 handling and the use of post-mortem material were approved by the Animal Welfare and Ethical
471 Review Body of Moredun Research Institute in accordance with the U.K. Animals (Scientific
472 Procedures) Act 1986.

473

474 **RNA extraction and sequencing.**

475 For RNA-Seq, RNA was extracted from frozen tissue samples from seven distinct sites of the
476 lungs of each animal and pooled in equal amounts. Total RNA from cryosectioned tissue was
477 extracted using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. RNA

478 concentration and purity were measured (ND-1000 Nanodrop) and RNA integrity was confirmed
479 (Agilent RNA 6000 Nano kit with Agilent 2100 Bioanalyzer). All samples had an RNA integrity
480 number (RIN) greater than 8.0. Prior to sequencing, frozen lung sections derived from the same
481 lung sites were examined by histology, which confirmed the presence of OPA tumor lesions in
482 the JSRV-infected animals and the absence of lesions in the mock-infected negative controls.
483 Total RNA was processed (TruSeq RNA Library kit) to generate cDNA libraries according to the
484 manufacturer's instructions and subsequently sequenced with an Illumina HiSeq2000 instrument
485 using 100 bp paired-end sequencing (Edinburgh Genomics, University of Edinburgh, UK).

486

487 **Processing of next generation sequencing data and differential expression analysis.**

488 Raw sequencing reads were processed to remove adaptors and poor-quality sequences (Q25 and
489 below) using Cutadapt 1.10 (63). Non-redundant reads were then mapped to the sheep
490 (Oar_v3.1, Ensembl FTP release 74) and JSRV (GenBank accession AF105220.1) genomes
491 using HISAT2-2.0.4 (64). The quantification of gene expression was calculated using HTSeq-
492 counts (65). Transcripts with fewer than 100 total reads across the eight samples were excluded.
493 The sheep annotation (GTF) was obtained from Ensembl (Oar_v3.1.79). The virus and sheep
494 gene counts were imported into the edgeR package (66) and counts were normalized using a
495 trimmed mean of M-values (67) and fitted to a negative binomial generalized log-linear model to
496 calculate the dispersion factor for each gene (68). Differentially expressed genes were then
497 identified by applying a FDR cutoff of 0.05 (69). Principal component analysis of normalized
498 counts was performed using only sheep gene expression (i.e., counts from viral genes were
499 removed) in order to see the variation between the two groups.

500

501 **Gene function annotation and pathway analysis.**

502 Identification of enriched KEGG pathways in the upregulated and downregulated gene lists was
503 performed with DAVID (Database for Annotation, Visualization, and Integrated Discovery) v6.8
504 (26). Pathway analysis was performed using Ingenuity Pathway Analysis (IPA), version 01-04
505 (27) to infer the functional roles and relationships of the differentially expressed genes based on
506 the log₂ fold-change value of each gene.

507

508 **Comparative analysis with human datasets.**

509 To compare significantly-expressed genes in JSRV-infected lung with data from human NSCLC,
510 HTSeq-counts from RNA-Seq data for LUAD and LUSC were obtained from the GDC portal
511 (portal.gdc.cancer.gov). 57 LUAD and 49 LUSC patients were selected for which corresponding
512 normal and tumor data were available. Differentially expressed genes in human tumors were
513 identified using the edgeR pipeline as for the sheep data. The list of differentially expressed
514 genes in human data was filtered for only those that have an orthologous gene in sheep
515 (identified using Ensembl Biomart) and were differentially expressed in sheep. The comparison
516 of expression of these short-listed genes was made using the correlation between expression
517 profile (i.e., fold-change) in humans and in sheep. The gene lists obtained are presented in
518 Supplementary Data S5.

519

520 **RT-qPCR**

521 RT-qPCR was performed according to MIQE recommendations (70). Primers and probes for
522 reference and target genes are summarized in Table 2. RT-qPCR was performed using an ABI
523 7000 Sequence Detection System in 96-well plates (Applied Biosystems) with either TaqMan

one-step RT-PCR reagents (Applied Biosystems; JSRV, CCL2) or Power SYBR-Green RNA-to CT 1-Step Kit (Applied Biosystems; all other target genes). Each sample (5 sites per animal) was tested in duplicate using 100 ng of RNA in a 20 µl final reaction volume. All experiments with SYBR-green included a melting curve analysis to confirm the specificity of the amplicons (95°C for 15 s, 60°C for 20 s and 95°C for 15 s). Standard curves constructed from 10-fold serial dilutions of positive control RNA were used to determine efficiency and replicate quality (R²) of each primer set. In addition, the level of gene expression in experimental samples was ensured to lay within the limits of the standard curve. For comparison of RNA transcription levels between samples, results from the RT-qPCR experiments were normalized to two reference genes, succinate dehydrogenase (SDHA) and β-actin (ACTB) (pre-determined as stable reference genes using geNORM (71)). Statistical analysis for quantitative PCR was performed by group-wise comparison based on PCR efficiencies and the mean crossing point deviation between the sample and control group using Relative Expression Software Tool (REST) (72).

538

539 **Immunohistochemistry and immunofluorescence**

Tissue sections were processed routinely through graded alcohols, embedded in paraffin-wax and IHC was performed on sections (4 µm) mounted on charged glass microscope slides as previously described (9). The dilutions and sources of the primary antibodies used are described in Table 3. Isotype controls were used in semi-serial tissue sections for each primary antibody. Additionally, the primary antibody was omitted to check for nonspecific labeling by the secondary antibody or the visualization system. Images for bright-field microscopy were examined using an Olympus BX51 microscope, and photographs were captured with an

Olympus DP70 camera with analySIS software (Soft Imaging System GmbH, Munster, Germany). Immunofluorescence was performed as previously described (9) using primary antibodies to AGR2 (1:100, Abcam ab76473), and JSRV Env (1:50) (73) with appropriate secondary antibodies conjugated with Alexa-488 (A11008, Molecular Probes) or Alexa-555 (A31622, Molecular Probes). Slides were mounted with medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories). Images were analyzed using a Zeiss Axio Imager 2 fluorescence microscope with Apotome, and AxioVision Software.

554

555 **Data Availability**

556 The raw RNA-Seq reads (fastq data) of each sample are present in the European Nucleotide
557 Archive with the accession ID PRJEB27638.

558

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569

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807 domestic sheep (*Ovis aries*). *PLoS Genet* 13:e1006997.
- 808

809

810 **Table 1 Read mapping statistics**

811

	Lamb ID ^a							
	Infected 1M_66days	Infected 2F_85days	Infected 3M_71days	Infected 4M_85days	Control 5M_66days	Control 6M_85days	Control 7M_71days	Control 8F_85days
Total reads	64,879,262	72,907,048	84,205,226	68,631,006	60,417,140	69,875,636	64,020,136	72,061,316
Total quality trimmed	63,766,561	71,678,589	82,667,841	67,438,835	59,431,332	68,705,449	62,964,748	70,830,855
Quality trimmed (%)	98.28%	98.32%	98.17%	98.26%	98.37%	98.33%	98.35%	98.29%
Total uniquely mapped	51,720,272	58,431,025	67,009,475	55,039,775	48,778,008	55,995,176	51,447,365	58,063,922
Uniquely mapped (%)	79.72%	80.14%	79.58%	80.20%	80.74%	80.14%	80.36%	80.58%
Viral reads	109,952	68,763	342,032	196,608	54 ^b	62 ^b	128 ^b	49 ^b
Viral reads (%)	0.17%	0.09%	0.41%	0.29%	0.00%	0.00%	0.00%	0.00%

812

813

814 ^a Lamb ID indicates the infection status, sex (male (M) or female (F)) and days post-inoculation
815 when culled.

816 ^b Reads mapping to the JSRV genome represented 0.09 – 0.41% of total reads in the four
817 infected lambs. A small number of reads mapping to JSRV (49 – 128 per sample) were also
818 detected in the tissue from mock-infected control lambs. All of these reads mapped to regions of
819 very high similarity between JSRV and enJSRV (data not shown) and so can be attributed to the
820 transcription of endogenous viruses in the samples studied.

821

822 **Table 2 Primers and probes used in this study**

Target ^a	Primer sequences (5'-3')	Conc. (nM)	Size (bp)	GenBank Accession No. (Reference)
ACTB	F: CTGAGCGCAAGTACTCCGTGT R: GCATTTCGCGTGGACGAT	300 300	125	NM_001009784 (74)
SDHA	F: CATCCACTACATGACGGAGCA R: ATCTTGCCATCTTCAGTTCTGCTA	200 200	90	AY970969 (74)
IFN- α	F: GCCTGGATCAGCAGCTCACTG R: CTCAAGACTTCTGCTCTGACAACCT	200 200	188	AY 802984 (75)
IFN- γ	F: TTCTTGAACGGCAGCTCTGAG R: TGGCGACAGGTCATTCATCA	300 300	127	X52640 (76)
CSF2	F: GATGGATGAAACAGTAGAAGTCG R: CAGCAGTCAAAGGGAATGAT	500 500	261	NM_001009805 (77)
TGF- β 1	F: GAACTGCTGTGTTTCGTCAGC R: GGTGTGCTGTTGTACAGG	500 500	169	NM_001009400 (77)
TNF- α	F: GCCCTGGTACGAACCCATCTA R: CGGCAGGTTGATCTCAGCAC	200 200	82	NM_001024860 (75)
IL-1 β	F: CCTAACTGGTACATCAGCACTTCTCA R: TCCATTCTGAAGTCAGTTATATCCTG	200 200	95	NM_001009465 (75)
IL-6	F: TCCAGAACGAGTTTGAGG R: CATCCGAATAGCTCTCAG	500 500	236	NM_001009392 (77)
IL-8	F: ACTGCGAAAAATTCAGAAATCATTGTTA R: CTTCAAAAATGCCTGCACAACCTTC	500 500	53	S74436 (78)
IL-10	F: AGCAAGGCGGTGGAGCAG R: GATGAAGATGTCAAACCTCACTCATGG	200 200	90	NM_001009327 (75)
IL-18	F: ACTGTTTCAGATAATGCACCCAG R: TTCTTACACTGCACAGAGATGGTTAC	200 200	100	NM_001009263 (75)
CCL2	F: GCTGTGATTTTCAAGACCATCCT R: GGCGTCCTGGACCCATT Probe: AAAGAGTTTTGTGCAGACCCCAACC	900 400 900	72	DY503036 (79, 80)

823

824 ^a Amplification conditions were 30 min 45°C, 10 min 95°C, 40 cycles of (15s 95°C, 1 min 60
825 °C), except for CSF2 (30 min 45°C, 10 min 95°C, 40 cycles of (20s 94°C, 30s 57°C, 30s 72°C)),
826 CXCL8 (30 min 45°C, 10 min 95°C, 40 cycles of (20s 94°C, 30s 55°C, 30s 72°C)), TGF-beta
827 (30 min 45°C, 10 min 95°C, 40 cycles of (20s 94°C, 30s 55°C, 30s 72°C)), IL-6 (30 min 45°C,
828 10 min 95°C, 40 cycles of (20s 94°C, 30s 52°C, 30s 72°C)) and CCL2 (30 min 45°C, 10 min
829 95°C, 40 cycles of (20s 95°C, 30s 58°C, 30s 60°C)).

830

831 **Table 3** Summary of antibodies used for IHC

832

Target Antigen	IHC Dilution	Antibody type ^a	Source
JSRV Env (SU)	1/200	Mouse mab	(73)
AGR2	1/200	Rabbit mab	Abcam, ab134167
AREG	1/200	Rabbit mab	Abcam, ab224350
MST1/2	1/5000	Rabbit pab	Abcam, ab87322
LATS1/2	1/2000	Rabbit pab	Abcam, ab70565
Total YAP1	1/100	Rabbit mab	Abcam, ab52771
Phosphorylated (s127) YAP1	1/300	Rabbit pab	Abcam, ab76252
CD68	1/150	Mouse mab	DAKO, M0718
CD163	1/200	Mouse mab	Bio-rad, MCA1853
LGMN	1/400	Mouse mab	Abcam, ab125286
HIF1A	1/50	Mouse mab	ThermoFisher MA1-16511

833

834 ^amab: monoclonal, pab; polyclonal

835

836 **FIGURE LEGENDS**

837

838 **Figure 1. Host gene expression is modified in JSRV-infected lung tissue.**

839 A. Principal component analysis was performed using read counts from JSRV-infected and
840 mock-infected (control) lambs for 15,149 genes mapping to the sheep genome. Each circle
841 represents a mock-infected lamb and each triangle indicates a JSRV-infected lamb. The values
842 on each axis (PC1 and PC2) represent the percentage of variance explained by each component.
843 The principal component 1 (PC1) separates the infected and mock samples into two clusters with
844 the highest variance of 63%. Within these clusters, there is greater variance in the JSRV-
845 infected sheep than the mock-infected sheep and this likely corresponds to the proportion of
846 tumor-affected tissue in the samples. For example, the lamb 'Infected_F_85days' is the closest
847 to the mock-infected cluster and had the lowest proportion of reads mapping to JSRV (see Table
848 1), while the samples furthest from the mock-infected animals had the highest proportion of viral
849 reads. B. The gene expression data visualized as a 2D scatter plot of the \log_2 ratio of expression
850 values between infected and mock-infected tissue (i.e., the fold-change) versus the mean
851 expression across all samples. Each dot represents one gene and the red color indicates the 1,971
852 genes identified as differentially expressed between the infected group and mock-infected
853 controls using a false discovery rate (FDR) < 0.05 . C. Hierarchical clustering based on
854 normalized gene counts of differentially expressed genes in samples derived from JSRV-treated
855 and mock-infected lambs (1237 were upregulated and 734 downregulated). D. Hierarchical
856 clustering of the 25 most significantly upregulated and downregulated genes.

857

858 **Figure 2. Main Diseases and Biofunctions associated with differentially expressed genes in**
859 **JSRV-infected sheep lung.**

860 The figure shows the Biofunction enrichment profiles identified by Ingenuity Pathway Analysis,
861 plotted by relative statistical significance. Significance values were calculated based on a right-
862 tailed Fisher's exact test and the $-\log(p\text{-value})$ is displayed on the horizontal axis of the bar chart.
863 The taller the bar, the more significant the pathway effect.

864

865 **Figure 3. Mechanistic network analysis predicted by Ingenuity Pathway Analysis.**

866 The figure shows mechanistic network analysis by IPA of genes differentially expressed in
867 experimentally-induced OPA compared to mock-infected lung. A. Neovascularization network. B.
868 Network for cell viability and tumor growth. The regulators are colored by their predicted
869 activation state: activated (orange) or inhibited (blue). Darker colors indicate higher scores. The
870 edges connecting the nodes are colored orange when leading to activation of the downstream
871 node, blue when leading to its inhibition, and yellow if the findings underlying the relationship
872 are inconsistent with the state of the downstream node. Pointed arrowheads indicate that the
873 downstream node is expected to be activated if the upstream node connected to it is activated,
874 while blunt arrowheads indicate that the downstream node is expected to be inhibited if the
875 upstream node that connects to it is activated.

876

877 **Figure 4. Immunohistochemical detection of AGR2 and AREG in JSRV-infected cells.**

878 Immunohistochemical labeling of sheep lung with antibodies to JSRV SU (A, B, C) and AGR2
879 (D, E, F). A and D, serial adjacent sections of mock-infected sheep lung. B and E, serial sections
880 of OPA lesions in the lung of a lamb experimentally-infected with JSRV. C and F, serial sections
881 of lung tissue from a natural case of OPA. Brown pigment indicates positive labeling. G, H, I, J,

immunofluorescence labeling of lung tissue of an experimentally-infected lamb. Panel H, AGR2 (green); panel I, JSRV (red). Panel G shows staining with DAPI (blue) used to visualize nuclei and panel J shows the merged image. K, L and M, IHC labeling with an antibody to AREG. K, mock-infected lung shows labeling of some epithelial cells (arrows) and macrophages (arrowheads). L, JSRV experimentally-infected lamb and M, naturally OPA-affected sheep lung show labeling of OPA lesions.

Figure 5. Immunohistochemical detection of Hippo pathway regulators in OPA-affected lung.

IHC was performed on sections of ovine lung tissue with antibodies to proteins involved in Hippo pathway signaling. Left column, mock-infected sheep lung; middle column, experimentally-infected lamb; right column, natural OPA. Brown pigment indicates positive labeling. A-C, total YAP1; D-F, phosphorylated (inactive) YAP1 (P-YAP1); G-I, MST1/2; J-L, LATS1/2. Note P-YAP expression is localized in the cytoplasm of tumor cells (panels E and F), whereas total YAP1 expression is shown the nuclei and the cytoplasm of tumor cells (panels B and C). Black arrows in panels B and C indicate examples of nuclear locating YAP1.

Figure 6. RT-qPCR detection of cytokine expression in OPA.

The figure shows box-whisker plots of relative expression of selected genes in OPA-affected lung tissue compared to healthy control tissue measured by RT-qPCR. A. Experimentally-induced OPA relative to lung tissue from age and sex matched mock-infected lambs. B. Natural OPA relative to healthy adult sheep lung. Data analysis was performed using the software tool REST (72), which calculates the significance of the expression ratio based on the use of two reference genes and the amplification efficiency for each gene. Significant changes in expression

906 are indicated by asterisks (*; standard error <0.005 at P=0.05). The dotted line within each box
907 represents the median value, the boxed area encompasses the interquartile range and the whiskers
908 indicate the maximum and minimum data points. Note the difference in scales on the Y axis
909 between the two charts. The data are consistent with the results of RNA-Seq analysis for these
910 genes in experimentally-infected lambs and controls, where *CSF2*, *CCL2*, *CXCL8* and *IL6* were
911 upregulated, *TNF* was downregulated and *IL1B*, *IL10*, *IL18* and *IFNG* were not significantly
912 changed. *TGFB1* and *IFNA* were not detected by RNA-Seq although *TGFB3* was upregulated.
913 (Note that *IFNA* was not examined in experimental OPA.)

914

915 **Figure 7. Expression of macrophage-related genes is altered in OPA.**

916 To identify macrophage-related functions in our data, a list of macrophage-related genes was
917 compiled based on Gene Ontology (GO) terms (891 genes) and those present in macrophage
918 coexpression clusters from the recently published sheep genome atlas (450 genes) (81) (see
919 Supplementary Dataset S3). Combining these two lists gave a total of 1255 genes (86 were
920 common to both sets), of which 1076 were present in our RNA-Seq data and 198 were
921 differentially expressed in JSRV-infected lung tissue. A. Hierarchical clustering of 198 markers
922 related to macrophage function that are differentially expressed in OPA. B. Hierarchical
923 clustering of the 25 most significantly upregulated and downregulated macrophage-related genes
924 in OPA. Orange coloring indicates upregulation, blue indicates downregulation.

925

926 **Figure 8. Immunohistochemical detection of macrophage markers in OPA-affected lung**
927 **tissue.**

928 Immunohistochemistry of sheep lung labeled with antibodies to markers of macrophages. Left
929 column, mock-infected lung; middle column, experimentally-infected lung; right hand column,

930 natural OPA-affected lung. Brown pigment indicates positive labeling. A-C, labeling with an
931 antibody to CD68. D-F, serial sections of tissues shown in panels A-C labeled with an antibody
932 to CD163. Note the difference in distribution of labeling with the two antibodies. G-I, labeling
933 with an antibody to LGMN; J-L, labeling with an antibody to HIF1A. Note that antibodies to
934 LGMN and HIF1A label some tumor cells in addition to macrophages. In addition, LGMN
935 exhibits a different pattern of labeling of tumor cells in experimental and natural OPA, where
936 experimental cases have intense cytoplasmic labeling and natural cases have prominent labeling
937 of the apical region.

938

939 **Figure 9. Comparison of gene expression in OPA and human lung cancer.**

940 Genes differentially expressed in experimentally-induced OPA were compared with genes
941 differentially expressed in human lung cancer, including clinical stages I - IV of LUAD and I -
942 III of LUSC. (LUSC stage IV was not analyzed as there were only two samples present in the
943 data.) A. Correlation plot comparing differentially expressed genes in OPA with the different
944 clinical stages of human LUAD and LUSC. The height of each bar shows the percentage of
945 differentially expressed sheep genes that have differentially expressed orthologues in the human
946 data set. The shading indicates the Pearson correlation coefficient between the log ratios of genes
947 in common between the two lists. The data confirm closer similarity of OPA to LUAD than
948 LUSC and to stage I LUAD in particular. B. The differentially expressed orthologous genes in
949 both sheep and human stage I LUAD were analyzed with Ingenuity Pathway Analysis software
950 and the figure shows the enriched Diseases and Biofunctions associated with those genes. C.
951 The plot shows the changes in gene expression in experimentally-induced OPA and human stage
952 I LUAD for the 50 most significantly changed sheep genes with deregulated human orthologues.
953 Note that 37 of the 50 genes show a similar direction of change in expression, whereas 13 show

954 divergent changes, highlighting similarities and differences between the sheep and human
955 diseases.

















